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Full Length Research Paper

Efficient production of transgenic soybean (*Glycine max* [L] Merrill) plants mediated via whisker-supersonic (WSS) method

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The present study was designed to evaluate the transformation efficiency and proof the capability of whisker supersonic (WSS) method as an alternative option for soybean (*Glycine max* [L] Merrill) transformation. We compared soybean transformation efficiency obtained by WSS-mediated with that of particle bombardment transformation by carrying out molecular analysis of the T_0 plants in two independent experiments. For this, we used for both transformation techniques the same genotype, the same plasmid and the same selection method. To assess the efficiency of soybean genetic transformation, we evaluated the efficiency of multi gene transformation by the selection with hygromycin and the expression of green fluorescent protein [*sGFP (S65T)]* resulted from both techniques. Regenerable embryogenic cells were induced from immature cotyledons of soybean c.v Jack on MSD40 media within 3 weeks then proliferated on FN lite liquid media and engineered with pUHG gene construct through both WSS and particle bombardment-mediated transformation. The pUHG was constructed with pUC 19 and contain the *hpt* gene conferring resistance to hygromycin as a selective marker and *sGFP(S65T)* as a reporter gene. Fluorescence microscopy screening after the selection of hygromycin, identified the clearly expression of *sGFP(S65T)* in the transformed soybean embryos. Stable integration of the transgenes was confirmed by polymerase chain reaction (PCR) and Southern blot analysis. The average transformation efficiency achieved with WSS was higher than that obtained by particle bombardment and hence it may represent an alternative method for soybean transformation.

Key words: *Glycine max*, whisker supersonic, particle bombardment, transgenic.

INTRODUCTION

Soybean is an economically important oil and protein crop for which *in vitro* technology has considerable potential (Santos et al., 1997). The development of a routine efficient transformation system has, therefore, been the subject of intensive research by scientists committed to genetic improvement of soybean in many laboratories. Genetic transformation of soybean has been reported by using various DNA delivery methods and plant tissues including microprojectile bombardment of shoot meristems (McCabe et al., 1988) and embryonic suspension culture (Finer and McMullen 1991; El-Shemy et al. 2002, El-Shemy et al., 2004; Khalafalla et al., 2005) and *Agrobacterium tumefaciens* mediated T-DNA delive-

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ry into immature cotyledons (Parrott et al., 1989; Yan et al., 2000), embryonic suspension culture and axillary meristematic tissue located in seedling cotyledonary-nodes (Hinchee et al., 1988). Despite some success, each of these methods has a serious drawback in term of time, cost and transformation efficiency. Consequently, the recovery of fertile soybean plants has proved to be more difficult and reports on successful genetic transformation remain scarce, and spontaneous rather than routine. Therefore, there is a great need to develop new efficient technique for soybean transformation.

The recently developed whisker supersonic (WSS) method for rice suspension culture cells transformation (Terakawa et al., 2005) may represent one of the alternative options for soybean suspension cell transformation that should be attempted. Whisker-supersonic (WSS) method is the direct transformation method recently developed at the Central Research Laboratories of the Hokko Chemical Industry Company in Japan. WSS-mediated gene transfer use whisker of potassium titanate fibers with an average diameter of 0.5 µm and length ranging from 3- 50 µm accompanied with supersonic treatment. The technology is elegant in its simplicity, very low start up cost and potential for scaling up, offer an attractive means of delivering DNA into genome of recipient cells.

DNA transfer via particle bombardment is currently the standards technique applied for soybean transformation (Khalafalla et al., 2005; El-Shemy et al., 2006). Therefore in this study in order to evaluate the transformation efficiency and proof the capability of whisker-supersonic (WSS) technique as an alternative option for soybean transformation, we propose to do a comparative assessment for soybean transformation frequencies obtained from both whisker-mediated transformation and particle bombardment in two independent experiments. For this, we used for both techniques the same genotype, the same plasmid and the same selection method. To assess the transformation efficiency, we evaluated the efficiency of multi gene transformation by the selection with hygromycin and the expression of *sGFP (S65T)* resulted from both techniques.

MATERIALS AND METHODS

Plasmid construction

The pUHG (SK) plasmid (6.5 kb) was a derivative of pUC19 and include a multiple cloning site from pBlue Script SK+ (Stratagene, La Jolla, CA, USA) that is located between sGFP(S65T) reporter gene (Chiu et al., 1996) and *hpt*, which encodes hygromycin phosphotransferase and confers resistance to hygromycin (Figure 1). Both of the genes were under regulatory control of the cauliflower mosaic virus (CaMV) 35S promoter and the poly-adenylation region from the nopaline synthase gene (NOST). The plasmid was purified by QIAGEN plasmid kit according to manufacture's instructions and re-dissolved in TE buffer.

Initiation and proliferation of embryogenic cultures

Plants of soybean, *cv. Jack,* were grown in soil in the glass-house controlled at 25°C under natural light condition. Developing green pods were obtained when the immature cotyledons were about 4-5 mm long. After sterilization of the pod surface with 70% ethanol followed with 3 sterile water rinses, the immature cotyledons were extracted, removed the end with the embryonic axis, and placed with adaxial side up on MSD40 medium in 90 mm disposable plastic petri dishes. The medium consisted of MS salts (Murashige and Skoog 1962) and B5 vitamins (Gamborg et al., 1968) that were supplemented with 3% sucrose and 2,4-dichlorophenoxyacetic acid (2,4-D, 40 mg ml^{-1}), adjusted to pH 7.0, and solidified with 0.2% Gelrite (Wako, Osaka, Japan). Somatic embryos were initiated at 25°C under cool white fluorescent light (23 h light, 1 h dark regime, 5 to 10 µmol m^{-2} s^{-1}) for 3 to 4 weeks. Embryogenic tissue was then suspended and maintained in FN Lite liquid medium, consisting of FN Lite macro salts, MS micro salts, and B5 vitamins supplemented with asparagine (1 g l^{-1}), 2,4-D (5 mg l^{-1}), and 1% sucrose and adjusted to pH 5.8. The cultures were maintained on a rotary shaker (100 rpm) and were sub cultured once a week into 25 ml of fresh FN Lite liquid medium in a 100-ml flask (El-Shemy et al., 2004; Khalafalla et al*.,* 2005).

Whisker-mediated transformation

Whisker Preparation and whisker transformation were optimized according to methods described previously by Terakawa et al. **(**2005). Whisker of potassium titanate fibers (Whisker LS20; TITAN KOGYO) with an average diameter of 0.5 µm and length ranging from 3- 50 µm were used to deliver the pUHG plasmid into soybean embryogenic tissues. About 50 mg of dry fibers into tube were sterilized with 1 ml of EtOH (100%) overnight and dried by opening the cap in the clean-bench. The sterilized whiskers were stored at 4°C until use. The dry whiskers were handled in an exhausted fume hood to avoid inhalation. Sterile whiskers in liquid medium (1% w/v) containing 1/3 MS basal salt and 30 g/l sucrose were mixed using a vortex mixer.

For whisker transformation (Figure 2) approximately 250 µl of embryogenic tissues were dispensed into 1.5 ml Eppendorf tube. The 1% (w/v) whisker suspension (500 µl), which was vortexed immediately before was added to this tube and tapped for mixing. After centrifugation at 3,000 rpm for 5 s, the supernatant was removed. Then plasmid pUHG (SK) (120 µl) was added to the tube and the tube was tapped strongly to mixing. Immediately the suspension was centrifuged for 5 min at 15,000 rpm at 4°C and tapped. This was repeated three times. After kept on ice for 10 min, the tube containing the mixer of cell suspension, whisker plasmid were subjected to supersonic treatment with disrupter for 1 min (Bioruptor UCD-200, COSMO BIO) at room temperature. Then, the mixture was washed with fresh liquid medium to remove as many whiskers as possible. The whisker-treated cells were then transferred into 35x10 mm plastic Petri plate for subculture.

Particle bombardment-mediated transformation

Particle bombardment transformation systems for soybean were optimized according to our previous method (Khalafalla et al., 2005; El-Shemy et al., 2004, 2006). Embryogenic tissue clumps (~0.8 g) were placed in the center of 90 mm plates containing MSD20 medium, consisting of MS salts and B5 vitamins supplemented with 3% sucrose, asparagine (1 g l^{-1}), and 2,4-D (20 mg l^{-1}) , adjusted to pH 5.8, and solidified with 0.2% Gelrite. Particle bombardment was performed with a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, Richmond, CA, USA). Plasmid DNA for particle bombardment was isolated with a Plasmid Midi Kit (Qiagen, Valen-

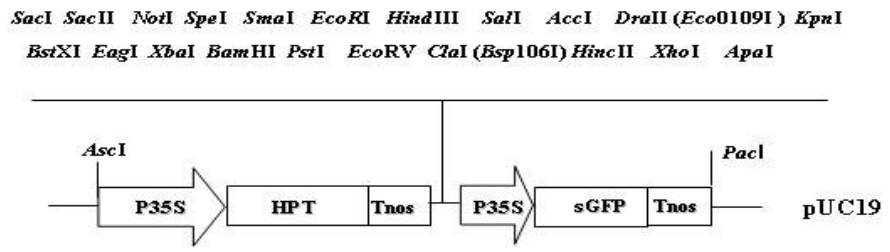

Figure 1. The expression plasmid pUHG(SK), used for soybean transformation. p35S, 35S promoter of cauliflower mosaic virus: *hpt*, hygromycin phosphotransferase gene ; T*nos*, 3' terminator sequence of nopaline synthase gene ; and *sGFP (S65T)* modefied green florescent protein gene. The position of unique restriction enzyme sites in the 6.5 kb vector are indicated.

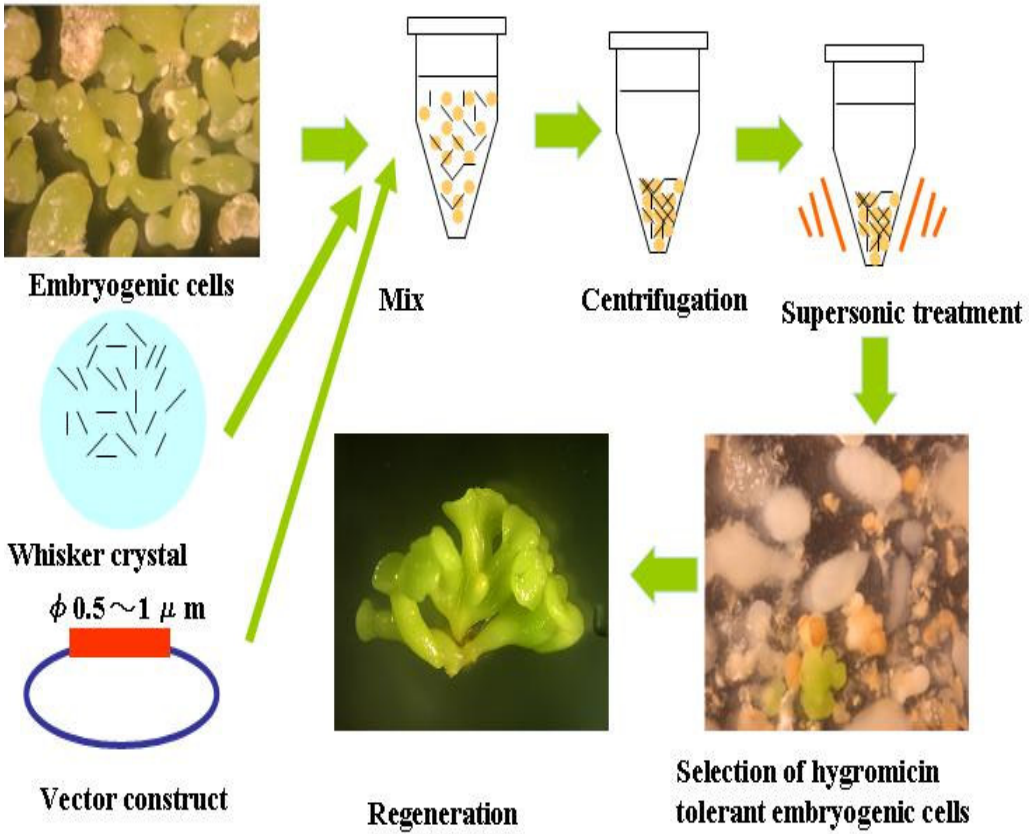

Figure 2. Scheme of whisker-mediated transformation of soybean.

cia, CA, USA) from *Escherichia coli* DH5α harboring pUHG, and aliquots (1 µg) of the purified DNA were precipitated onto 0.5 mg of 1.0-µm diameter gold particles by the $CaCl_2$-spermidine method (Klein et al., 1988). Each plate of embryogenic tissue was bombarded twice at a distance of 6 cm and a pressure of 1100 psi.

Selection and plant regeneration

Whisker-treated and the bombarded embryogenic tissues were resuspended in the FN Lite medium. One week later tissues were transferred in fresh FN Lite medium containing 15 mg/L hygromycin B (Roche Diagnostics, Mannheim, Germany). The tissues were transferred in fresh antibiotic-containing FN Lite medium weekly for 3 additional weeks. After then the white lumps of tissues that contained blight green lobes of embryogenic tissues were selected and transferred in fresh FN Lite medium containing 30 mg/L hygromycin B. The hygromycin tolerant tissues were selected and resuspended in fresh antibiotic-containing FN Lite medium weekly for 3 additional weeks. Hygromycin tolerant embryos were resuspended in FNL0S3S3 liquid medium, which contained FN Lite macro salts, MS micro salts and B5 vitamins supplemented with 1 g/L asparagine, 3% sucrose, and 3% sorbitol (pH 5.8). Three weeks after suspension, excess liquid of the developing embryos was

Table 1. Soybean transformation efficiency via whisker supersonic (WSS) and particle bombardment (PB).

Construct	Method of DNA delivery	Number of plates	Number of plants selected with hygromycin {PCR hpt+}	Number of plants expressed sGFP (s65T){PCR hpt and gfp+} (plates)
pUHG(SK)	WSS	11	67	26(5)
pUHG(SK)	PB	15	90	12(4)

withdrawn with sterile filter paper, and the embryos were placed in dry petri dishes for 3 to 5 days. After the desiccation treatment, the embryos were placed on MS0 medium containing MS salts, B5 vitamins, 3% sucrose, and 0.2% Gelrite (pH5.8). The germinating plantlets were transferred to 1/2 B5 medium. After root and shoot elongation, plantlets were transferred to pots containing soil, and maintained under high humidity. Plantlets were gradually adapted to ambient humidity and placed in the glass-house**.**

GFP detection

The expression of *sGFP (S65T)* was observed in embryogenic tissue, mature embryos, and regenerated plants with a stereomicroscope (Leica Microsystems, Wetzlar, Germany) equipped with a filter set for excitation between 455 and 490 nm and emission above 515 nm (Chiu et al., 1996).

Polymerase chain reaction (PCR)

DNA was extracted from the leaves of the putative transgenic T_0 plants by using Kurbo PI-50α machine (Kurabo industries, LTD. Biomedical Department, Osaka, Japan) according to the plant DNA ver.2 method. PCR analysis was conducted to screen transformed plants in a 20 µl reaction mixture containing 10 ng of genomic DNA, 200 µM of each of dNTP, 0.2 µl of each primer, and 2.5 units of Ampli-taq Gold polymerase (Applied Biosystems, Foster City, CA USA) in the corresponding buffer. Reactions were hot-started (9 min at 94°C) and subjected to 30 cycles as fellows: 30 s at 94°C; 1 min at 55°C; and 1 min at 72°C. The last extension phase was prolonged 7 min at 72°C. The primers set for *hpt* was designed for amplification of the 560 bp fragment; sequences are 5'-ATCCTTCGCAAGACCCTTCCT-3' and 5'-GGTGTCGTCCATCACAGTTTG-3' (*hpt*), and the primer set for sGFP(S65T) was designed for amplification of the 708 bp fragment; sequences are 5' AGGTACCGGATCCCCCCTCAGAA-3' and 5'-AGAGCTCCGATCTAGTAACATAGATGACACC -3'.

Southern blot analysis

Total genomic DNA was isolated from both untransformed control plantlets and transformed plantlets by the method of Murray and Thompson (Murray and Thompson, 1980). Southern blot analysis was conducted to confirm stable integration of transgenes. 10 µg of total DNA was digested with the restriction enzyme, *HindIII*, and digested DNA was separated by electrophoresis through a 1% agarose gel and then transferred onto hybond N+ membrane (Amersham Biosciences, Buckinghamshire, England). Labeling of probe and detection of hybridization were conducted following the protocol of ECL direct nucleic acid labeling and detection (Amersham Biosciences). DNA fragments corresponding to the *sGFP(S65T)* and *hpt* genes were amplified from the pUHG (SK) plasmid by same primer sets for the PCR analysis, and used as hybridization probe on Southern blot membranes.

RESULTS AND DISCUSSION

The delivery of transgenes into embryonic tissues by particle bombardment is commonly used in soybean transformation (Khalafalla et al*.,* 2005; El-Shemy et al., 2004, 2006). In order to evaluate the transformation efficiency and proof the capability of whisker supersonic (WSS) as an alternative option for soybean transformation we carried out a comparative assessment for its transformation efficiency with that obtained from particle bombardment using two independent experiments. For both experiments, the same genotype, the same plasmid and the same tissue culture system including selection method were used. Dai et al. (2001) reported that to minimize the influence of uncontrollable factors during development of transgenic plants by two different techniques, the same genotype, the same batch of calli, the same plasmid and the same experimentation must be used.

The result for evaluation of transformation efficiency obtained with pUHG construct for whisker and particle bombardment presented in Table 1. Hygromycin-resistant transgenic lines developed from both methods were confirmed by PCR analysis of the *hpt* gene. Whisker-treated and bombarded embryogenic tissue with the pUHG (SK) construct resulted in expression of *sGFP (S65T)* that was detectable with a fluorescence microscope within 6 h. The expression was maximal after one day, subsequently both the level of expression and the number of foci expressing GFP decreased (data not Shown), Similarly El- Shemy et al. (2004) were detected same observation. There were no significant differences for *sGFP (S65T)* expression and the number of foci expressing *sGFP (S65T)* between whisker and particle bombardment-mediated transformation (data not shown). *SGFP (S65T)* as a reporter gene may be useful to increase the transformation efficiency of transgenic soybean with avoiding gene co-suppression (El-Shemy et al., 2006).

After 6 weeks of selection with hygromycin, some green clumps of tissue were found to express *sGFP (S65T)*, indicating that they contained stably transformed cells. The hygromycin-tolerant tissues were allowed to mature in FNL0S3S3 liquid medium and the resulting embryos were germinated on MS0 medium. The presence or absence of *hpt* in all rooting plants obtained by whisker or particle bombardment was determined by PCR analysis. The whisker method showed higher trans-

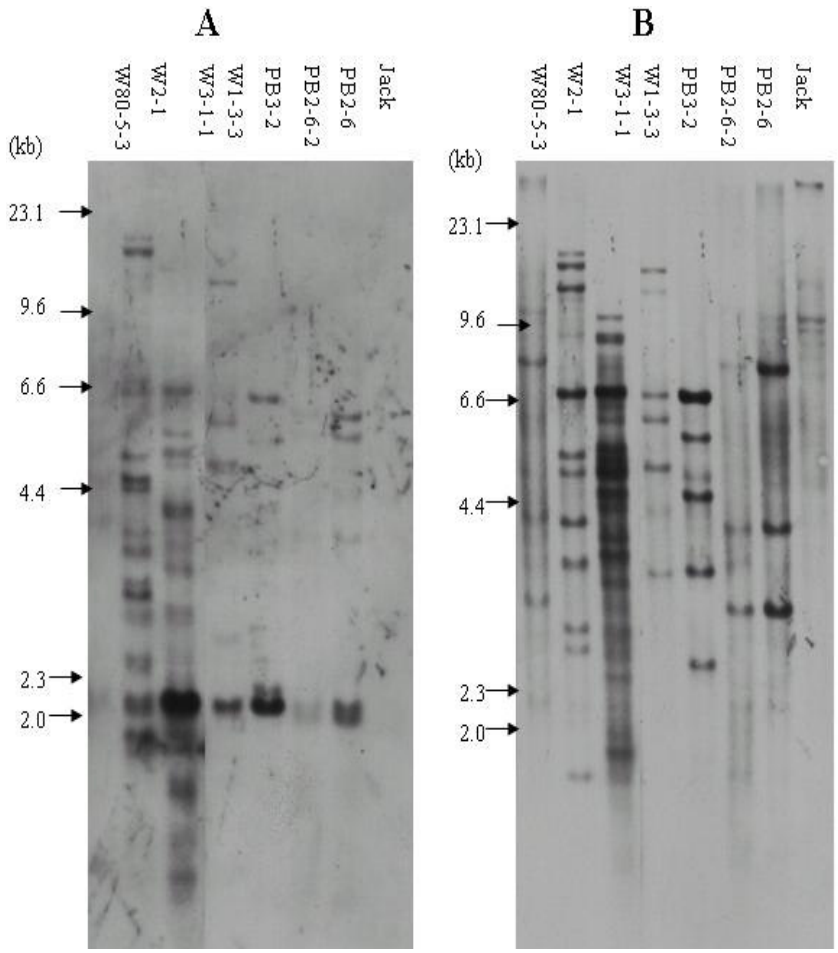

Figure 3. Southern blot analysis of individual T_0 plant transformed with pUHG (SK). Total DNA from a nontransformed control plant (*jack*) or from transgenic plants generated by particle bombaedment (PB) and wisker supersonic (W) was digested with Hind III and subjected to southern hybridization with probes specific for hpt (A) or for sGFP(s65T) (B). Arrows indicate the positions of the markers.

formation efficiency compared to that obtained by particle bombardment, where 26 transgenic plants obtained from 5 different plates by whisker compared only to 12 plants obtained from 4 different plates for particle bombardment (Table 1).

In addition to transformation frequency, other factor must be taken into account when considering different gene transfer methods, such as the ease of use and the cost, in this case whisker supersonic in addition to simplicity it has a very low start up cost (Terakawa et al., 2005) compared to particle bombardment, which is use an expensive apparatus.

We performed southern blot analysis to confirm the stable integration and estimate the copy number of transgenes in 3 and 4 transgenic plants obtained by particle bombardment and whisker supersonic, respectively. Total genomic DNA isolated from transgenic plants ascertained the presence of all transgene cassettes by PCR, was digested with *Hind III* and hybridized with one of the two probes for *hpt*, and *sGFP(S65T)* (Figure 3). All transgenic plants obtained from both direct transformation techniques showed multi copy hybridization bands with different bandings pattern for both gene cassettes. These results suggest that genetic rearrangement or fragmention of the vector had occurred in some of the transgenic plants. We have previously reported similar results for both gene cassettes (Khalafalla et al*.,* 2005). The fact that, the direct DNA delivery systems tend to result in integration of multiple copies of transgenes at single loci and rearrangement of transgenes was reported by many reporters (Finnegan and McElroy, 1994; Flavell 1994; Pawlowski and Somers, 1996; Kohli et al., 1998; El-Shemy et al., 2004). Although our results showed that WSS resulted in transgenic plants containing higher copy number of bands for both genes than particle bombardment, the transient *sGFP(S65T)* expression is similar for both techniques. Dean et al. (1988) and Hobbs et al. (1993) reported that there was no clear correlation between transgene expression and transgene copy num-

ber. The effects of transgenic copy number on the level of gene expression are known to be complex. Though it was anticipated that the increase of transgene copy number would increase the expression level (Dai et al., 2001; El-Shemy et al., 2004; El-Shemy et al., 2006).

The development of a fast procedure of direct plant transformation is the main result achieved in this study. Comparison of data confirms that the whisker supersonic technique is efficient than particle bombardment and therefore, represent an alternative option for soybean embryogenic tissue transformation hence, increases the possibility for introducing useful traits into soybean. To our knowledge, this is the first attempt for using whisker technique in producing soybean transgenic plants.

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REFERENCE

Chiu WL, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996). Engineered GFP as a vital reporter in plants. Current Biol. 6: 325-330.

Dai S, Zheng P, Marmey P, Zhang S, Tian W, Chen S, Beachy RN, Fauquet C (2001). Comparative analysis of transgenic rice plants obtained by Agrobacterium-mediated transformation and particle bombardment. Mol. Breed. 7: 25-33.

Dean C, Jones J, Favreau M, Dunsmuir P, hedbrook J (1988). Influence of flanking sequences on variability in expression levels of an introduced gene in trangenic tobacco plants. Nucl. Acids Res. 16: 9267–9283.

El-Shemy HA, Khalafalla MM, Wakasa K, Ishimoto M (2002). Reproducible transformation in two grain legumes –soybean and azuki bean – using different systems. Cellular & Mole. Biol. Lett. 7:709 – 719.

El-Shemy HA, Teraishi M, Khalafalla MM, Katsube-Tanaka T, Utsumi S, Ishimoto M (2004). Isolation of soybean plants with stable transgene expression by visual selection based on green fluorescent protein Molecular Breeding 14: 227–238, 2004.

El-Shemy HA, Khalafalla MM, Fujita K, Ishimoto M (2006). Molecular control of gene co-suppression of transgenic soybean. J. Biochem. and Mole. Biol. 39: 61-67.

Finer JJ, McMullen MD (1991). Transformation of soybean via particle bombardment of embryogenic suspension culture tissue. *In vitro* Cell and Develop Biol - Plant 27P: 175-182.

Finnegan J, McElroy D (1994). Transgene inactivation: plants fight back. Bio/technology 12: 883–888.

Flavell RB (1994). Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc. Natl. Acad. Sci. USA 91: 3490–3496.

Gamborg OL, Miller RA, Ojima K (1968). Nutrient requirments of suspension cultures of soybean root cell. Exp. Cell Res. 50: 151-158.

Hinchee MAW, Conner-Ward DV, Newell CA, McDonnell RE, Sato SJ, Gasser CS, Fischhoff DA, Re D, Fraley RT, Horsch RB (1988). Production of transgenic soybean plants using Agrobacterium-mediated DNA transfer. BioTechnol. 6:915-922.

Hobbs SLA, Warkentin TD, DeLong CMO (1993). Transgene copy number can be positively or negatively associated with transgene expression. Plant Mol. Biol. 21: 17–26.

Khalafalla MM , Rahman SM, El-Shemy HA, Nakamoto Y, Wakasa K, Ishimoto M (2005). Optimization of particle bombardment conditions by monitoring of transient sGFP(S65T) expression in transformed soybean. Breeding Sci. 55:257-263.

Klein TM, Gradziel T, From ME, Sanford JC (1988). Factors influencing gene delivery into *Zea mays* cells by high-velocity microprojectiles. Biotechnol. 6: 559–563.

Kohli A, Leech M, Vain P, Laurie DA, Christou P (1998). Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot spots. Proc. Natl. Acad. Sci. USA 95: 7203–7208.

McCabe DE, Swain WF, Martinell BJ, Christou P (1988). Stable transformation of soybean (*Glycine max*) by particle acceleration. BioTechnol. 6: 923-926.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 473-497.

Murray MG, Thompson WF (1980). Rapid isolation of high molecular weight DNA. Nucleic Acids Res. 8: 4321-4325.

Parrott WA, Hoffman LM, Hildebrand DF, Williams EG, Collins GB (1989) Recovery of primary transformants of soybean. Plant cell rep 7:615-617.

Pawlowski WP, Somers DA (1996). Transgene inheritance in plants genetically engineered by microprojectile bombardment. Mol. Biotechnol. 6: 17–30.

Santos KGB, Mundstock E, Bodanese-Zanettini MH (1997). Genotype specific normalization of soybean somatic embryogenesis through the use of an ethylene inhibitor Plant Cell Reports 16: 859–864.

Terakawa T, Hisakazu H, Masanori Y (2005). Efficient whisker-mediated gene transformation in a combination with supersonic treatment. Breeding science 55: 456-358.

Yan B, Srinivasa RMS, Collins GB, Dinkins RD (2000). Agrobacterium tumefaciens-mediated transformation of soybean [Glycine max (L.) Merrill.] using immature zygotic cotyledon explants. Plant Cell Rep. 19:1090-1097.